Circulating DNA is a Useful Prognostic Factor in Patients with Advanced Non-small Cell Lung Cancer

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Background: Circulating DNA is observed at higher concentrations in patients with lung cancer than in controls. Qualitative and quantitative analysis of circulating DNA is a promising noninvasive tool. Our aim was to prospectively study the association between the catalytic subunit of telomerase (human telomerase reverse transcriptase [*hTERT*]) in plasma and clinical variables and survival in a large-scale non-small cell lung cancer (NSCLC) study.

Methods: Four hundred forty-six patients with stages IIIB and IV NSCLC with a median follow-up of 9.7 months (range, 0.5–45) were analyzed. Blood samples were collected before therapy start (cisplatin/docetaxel). Quantification of baseline circulating DNA was determined as the amount of free *hTERT* in plasma, by using real-time quantitative polymerase chain reaction.

Results: Patients with *hTERT* ≤49.8 ng/ml (median value) had a median time to progression (TTP) of 6.3 months compared with 4.9 for *hTERT* more than 49.8 ng/ml ($p = 0.001$). Overall survival (OS) was significantly higher (10.9 versus 9.3 months) at lower *hTERT* levels ($p = 0.012$). When calculations were done using *hTERT* as continuous variable, we did not observe independent significant differences. Thus, there is an apparent discrepancy in p values when *hTERT* is considered as a continuous versus dichotomized variable. There was a tendency to differentiate median *hTERT* levels with respect to response rates (complete response + partial response: 33.1 versus stable disease + progressive disease: 50.7 ng/ml, $p = 0.12$), but other clinical variables such as age, gender, performance status, stage, histology, and number of metastatic locations were not associated with *hTERT*. In multivariate analysis, *hTERT* was an independent prognostic variable for both TTP (hazard ratio: 1.44, $p < 0.001$) and OS (hazard ratio: 1.33, $p = 0.007$).

Conclusions: In advanced NSCLC, high pretreatment circulating *hTERT* level is an independent poor prognostic marker for TTP and OS. Circulating DNA is a noninvasive marker, which may help to improve the prognostic profile of these patients.

Key Words: *hTERT*, Telomerase, Plasma, Prognostic factors, Non-small cell lung cancer, NSCLC.

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During carcinogenesis, pathologic tissue liberates cells and cell components to the blood stream, even at early stages of the disease.[1–3] The presence of nucleic acids in blood was reported more than 30 years ago by Leon et al.[4] In the late 1990s, Kopreski et al.[5,6] found that patients with cancer have more free DNA and RNA in the plasma or serum than healthy subjects.

The origin of this genetic material was not well established, but research up to now has confirmed a high degree of correlation between the genetic changes in the tumor and those detected in the blood,[7–9] which supports that much of the circulating DNA in the patients with cancer derives from the tumor.[10] These observations laid the foundation for the development of cell-free DNA/RNA assays for early detection of cancer and for aid in diagnosis,[11–13] although critical voices have been raised against its relevance for screening and diagnostics.[14,15]

During cancer, however, determination of plasma- or serum-free DNA may be an important prognostic factor with respect to treatment and posttherapy follow-up.[16,17] The amount of free human telomerase reverse transcriptase (*hTERT*) in plasma serves as a surrogate of circulating DNA. This test is easily and noninvasively performed at any time during therapy or follow-up and requires only a limited blood sample. Nevertheless, the prognostic role has been questioned because some studies demonstrate an association with survival,[18,19] whereas others do not.[16,20,21]

Although the levels of circulating cell-free DNA in plasma or serum are higher in patients with lung cancer than in healthy controls, hitherto it is not resolved whether this will be of diagnostic or prognostic value. Neither has the association

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with clinicopathological variables such as age, gender, performance status, and stage of disease been established.

The objective of our study was to quantify the amount of the *hTERT* gene in cell-free DNA in plasma from a large-scale cohort of patients with non-small cell lung cancer (NSCLC) and to correlate *hTERT* with time to progression (TTP) and overall survival (OS), and clinicopathological variables.

MATERIALS AND METHODS

Patients

This study has been performed with blood samples from 446 patients diagnosed with advanced stage (IIIB–IV) NSCLC who were enrolled in a multicenter clinical trial of the Spanish Lung Cancer Group between February 2003 and January 2005. The patients were chemonaive at inclusion. Blood samples were obtained before the planned start of combination chemotherapy with cisplatin and docetaxel. The institutional ethical review board approved the study.

Sample Collection and DNA Isolation

All patients were sampled before chemotherapy; 10-ml samples of peripheral blood were collected in vacutainer tubes containing ethylenediaminetetraacetic acid as anticoagulant (Becton Dickson, NJ) for plasma recovery. Tubes were sent to the reference laboratory, and in less than 24 hours, ethylenediaminetetraacetic acid-blood samples were subjected to two centrifugation steps: an initial centrifugation for 10 minutes at 1100*g* at room temperature and a second centrifugation of the supernatants for 10 minutes at 2000*g* at room temperature. This plasma was stored at −80°C until further analysis.

Free DNA was extracted and purified from 400 μl of plasma by using commercial DNA affinity columns (QIAamp-Blood Mini Kit, QIAGEN, Chatsworth, CA), following the manufacturer's recommendations, and eluted in 60 μl of kit's elution buffer.

DNA Quantification in Plasma

The plasma-free DNA concentration was measured by using a previously described highly reproducible quantitative real-time polymerase chain reaction targeting the *hTERT* gene.[22] The two primers and the minor groove binding probe were designed to amplify specifically the gene of interest, *hTERT*, generating an amplicon of 98 base pairs. The sequences, synthesized by Applied Biosystems, Foster City, CA were as follows:

Forward primer: 5′ GGC ACA CGT GGC TTT TCG-3′
Reverse primer: 5′ GGA GAG CAG AGG CAG AGA TCA-3′
Minor groove binding probe: 6-FAM- 5′ TCC ACT CGA CGT CCT GA-3′

The reaction was done in 96-well plates with a total volume of 50 μl/well, as described previously.[22] A standard curve using genomic human DNA from 50 pg to 50 ng (Applied Biosystems) was added to every amplification plate as described previously.[22]

Statistical Analysis

hTERT was analyzed as a continuous and dichotomized variable. For the dichotomized *hTERT* variable, the cutoff level for low and high values was set at the median. The Kolgomorov-Smirnov test was used to examine whether a continuous *hTERT* variable or its transformations followed a normal distribution. A Martingale residual analysis was run to check whether there was a linear trend between *hTERT* levels and survival. TTP and survival were also compared with tercils of *hTERT* concentrations. As *hTERT* values did not show a normal distribution, the comparisons were done with the nonparametric Mann-Whitney *U* test and Kruskal-Wallis test and the Spearman nonparametric correlation analysis. OS was calculated from the time of diagnosis and TTP from the date of treatment start. OS and TTP were calculated using the Kaplan-Meier model, and differences between groups were assessed by the log-rank test. For the multivariate analysis, we used Cox-regression analysis. All statistically significant variables from the univariate analyses were entered into the multivariate Cox analysis. A p value below 0.05 was considered statistically significant.

RESULTS

Table 1 lists the detailed demographic and clinicopathologic characteristics of the patient population. The median age

TABLE 1. Patient Characteristics, Time to Progression, and Survival of 446 Patients with Advanced NSCLC

			Time to Progression		Overall Survival	
Variables	***n***	**Percentage**	**Median (mo)**	***p***	**Median (mo)**	***p***
Age (yr)						
Median (range)	60 (31–80)			0.17		0.39
≤60 yr	228	51	5.3		9.7	
>60 yr	218	49	5.9		10.8	
Gender						
Male	375	84	5.3	0.010	9.7	0.018
Female	71	16	6.7		13.5	
ECOG PS						
0	113	25	6.6	0.025	13.1	0.001
1–2	331	75	5.3		9.2	
Missing	2					
Histology						
Adenocarcinoma	222	50	5.6	0.30	9.9	0.69
Squamous carcinoma	139	31	5.6		10.8	
Large cell carcinoma	69	15	5.8		9.7	
Undifferentiated	16	4	4.2		7.7	
Stage						
IIIB	70	16	5.5	0.26	10.2	0.86
IV	376	84	5.6		10.0	
hTERT						
≤49.8 ng/ml	223	50	6.3	0.001	10.9	0.012
>49.8 ng/ml	223	50	4.9		9.3	

NSCLC, non-small cell lung cancer; ECOG PS, Eastern Cooperative Oncology Group performance stage.

was 60 years, and 84% of the patients were men. The majority of patients was in performance stage 1 (performance stage 1: 74%, performance stage 2: 1%), 50% had adenocarcinoma, whereas 84% had stage IV NSCLC. Objective response was seen in 27% (complete response: 0.7%, partial response: 26%), whereas 73% had stable or progressive disease. TTP and OS with respect to the clinicopathological variables are also presented in Table 1.

hTERT levels ranged widely from 0.8 to 43,735 ng/ml. The median for the whole patient population was 49.8 ng/ml. The continuous *hTERT* variable did not follow a normal distribution even after a square root transformation. Using the Martingale residuals analysis, there was no linear trend between *hTERT* levels and survival, indicating that the median value as cutoff may not be justified.

There were no significant associations between plasma *hTERT* concentrations and clinical characteristics such as age ($p = 0.61$), performance status (PS, $p = 0.48$), gender ($p = 0.51$), stage ($p = 0.84$), histology ($p = 0.23$), and number of metastatic locations ($p = 0.25$). There was a significant association between plasma *hTERT* concentrations and response to treatment ($p = 0.043$, Table 2). Complete response + partial response patients (33.1 ng/ml) had a significantly lower median pretreatment *hTERT* level, when compared with progressive disease (63.7 ng/ml, $p = 0.036$) but not stable disease patients (45.3, $p = 0.52$, Table 2). Progressive disease patients, however, had significantly higher *hTERT* levels than stable disease patients ($p = 0.035$, Table 2).

Using dichotomized *hTERT* levels TTP was significantly associated with plasma *hTERT*. Patients with pretreatment *hTERT* concentration ≤49.8 ng/ml had a median TTP of 6.3 months, whereas those more than 49.8 ng/ml had median TTP of 4.9 months ($p = 0.001$, Figure 1). When the material was divided according to tercils of *hTERT* plasma concentrations, TTP was 7.0, 4.7, and 5.3 months at first, second, and third tercils, respectively ($p = 0.003$). Patients with *hTERT* ≤49.8 ng/ml had a median OS of 10.9 months, whereas those more than 49.8 ng/ml had a median OS of 9.3 months ($p = 0.012$, Figure 2). According to tercils, median survival was 12.7, 9.6, and 9.8 months at first, second, and third tercils, respectively ($p = 0.013$).

To identify significant individual prognostic markers, Cox regression analysis was performed. Using the dichotomized *hTERT* variable, multivariate analysis revealed that plasma *hTERT* level (hazard ratio [HR]: 1.44, 95% confidence interval [CI]: 1.18–1.75, $p < 0.001$), Eastern Cooperative Oncology Group PS (HR: 1.31, 95% CI: 1.04–1.64, $p = 0.017$), and gender (HR: 1.46, 95% CI: 1.11–1.91, $p = 0.005$) had an independent prognostic role (Table 2). With respect to OS, plasma *hTERT* level (HR: 1.33, 95% CI: 1.08–1.63, $p = 0.007$), Eastern Cooperative Oncology Group PS (HR: 1.56, 95% CI: 1.22–1.99, $p < 0.001$), and gender (HR: 1.51, 95% CI: 1.12–2.02, $p = 0.004$) were independent prognosticators (Table 3). When square root transformed continuous *hTERT* variable was entered in the Cox regression analysis, *hTERT*

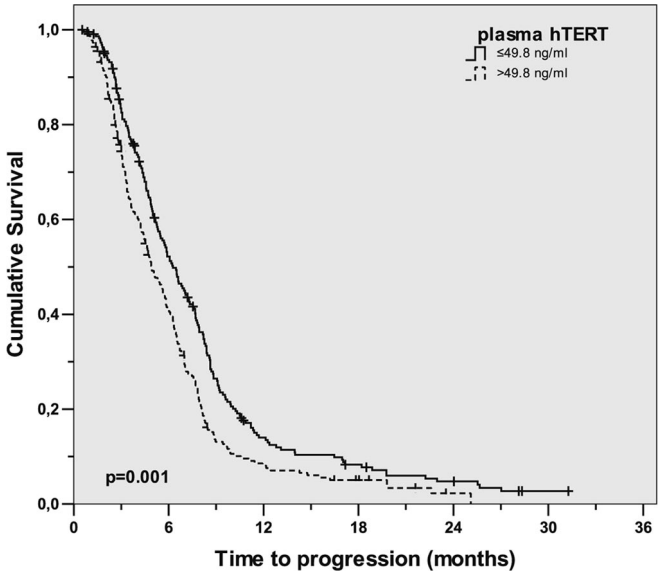

FIGURE 1. Time to progression according to pretreatment median *hTERT* plasma levels.

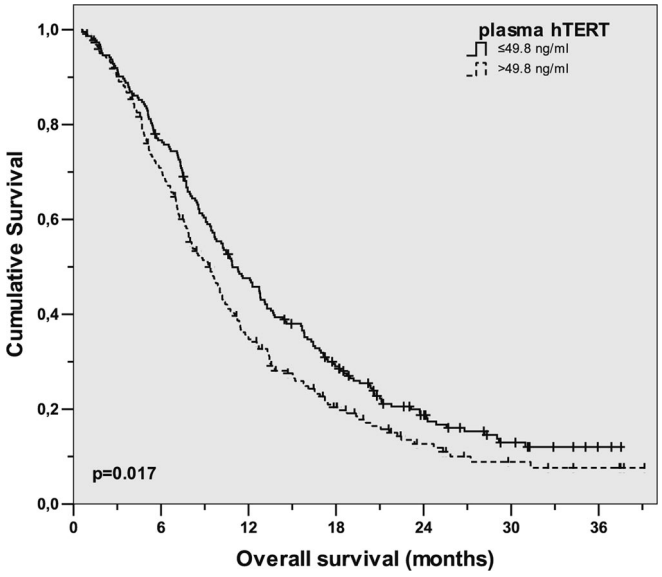

FIGURE 2. Overall survival according to pretreatment median *hTERT* plasma levels.

TABLE 2. *hTERT* Plasma Levels According to Objective Response Rates

	n	Median (ng/ml)	Mean (ng/ml)	Range (ng/ml)	p^a	p^b
CR + PR	110	33.1	219	1.2–3172.5	0.043	
SD	145	45.3	385	2.2–9648.3		0.52[c]; 0.035[d]
PD	155	63.7	632	0.8–43735.2		0.036[e]

[a] Kruskal-Wallis test.
[b] Mann-Whitney *U* test.
[c] CR + PR vs. SD.
[d] SD vs. PD.
[e] CR + PR vs. PD.
CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

TABLE 3. Results of Cox Regression Analysis Summarizing Significant Independent Prognostic Factors Regarding Time to Progression (TTP)

Factors	Hazard Ratio	95% CI	*p*
Gender			
Female	1.00		0.005
Male	1.46	1.11–1.91	
hTERT			
≤49.8 ng/ml	1.00		<0.001
>49.8 ng/ml	1.44	1.18–1.75	
ECOG PS			
0	1.00		0.017
1–2	1.31	1.04–1.64	

CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance stage.

TABLE 4. Results of Cox Regression Analysis Summarizing Significant Independent Prognostic Factors with Respect to Overall Survival

Factor	Hazard Ratio	95% CI	*p*
ECOG PS			
0	1.00		<0.001
1–2	1.56	1.22–1.99	
Gender			
Female	1.00		0.004
Male	1.51	1.12–2.02	
hTERT			
≤49.8 ng/ml	1.00		0.007
>49.8 ng/ml	1.33	1.08–1.63	

CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance stage.

was not an independent prognosticator for survival (HR: 1.003, 95% CI: 0.99–1.009, $p = 0.30$).

DISCUSSION

In 446 patients with advanced NSCLC, we investigated the prognostic role of circulating DNA (*hTERT*) in plasma sampled immediately before chemotherapy start. It was observed that plasma *hTERT* concentrations were significantly associated with the objective response rates. Using the median value as cutoff level, we found *hTERT* to be a significant independent prognostic factor for both TTP and OS. Nevertheless, this could not be reproduced when the continuous *hTERT* variable was used.

The strength of this study is the population size, in contrast to previous studies where the investigated cohorts, in general, have numbered 100 individuals or less. The lack of healthy control samples may be a weakness but of less importance in established advanced NSCLC than for screening purposes in healthy individuals.

Although the presence of nucleic acids in blood was reported more than 30 years ago,[4] the interest were significantly aroused at the publication in 2003 by Sozzi et al.[13,23] of plasma *hTERT* data from 100 patients with NSCLC and 100 age-, sex-, and smoking-matched controls. They found significantly higher levels of free circulating DNA in patients with NSCLC than in disease-free heavy smokers and concluded that this method should be used as a new approach for early detection of lung cancer.

Research into the role of circulating DNA in established cancers has demonstrated a strong power to discriminate patients with lung cancer from those with benign lung diseases or healthy individuals.[24,25] It has further confirmed a high degree of correlation between genetic changes in tumors and those detected in the blood.[10,15,26–29] These findings support the hypothesis that a significant portion of the circulating DNA in patients with cancer are derived from the tumor. In a thorough review, Fleischhacker and Schmidt[10] concluded that because these free circulating DNAs are a reflexion of the physiological and pathologic processes in the human body, it makes them a very interesting target for the development of clinically useful assays.

In several nonlung malignancies such as colorectal, breast, and prostate cancer, enhanced blood DNA levels have been shown to correlate with prognosis.[29–31] In NSCLC, there are conflicting data concerning the prognostic role of circulating *hTERT* in these patients. Three clinical studies,[16,20,21] of which the study by Sozzi et al.[16] is the largest with 84 patients with NSCLC, did now show an association with survival. Ludovini et al.,[21] in 76 operable stage I to III patients with NSCLC, found that circulating DNA declined after surgery and increased at recurrence, even though it did not show any prognostic significance.

In this study, we observed that high *hTERT* levels were significant and independent prognosticators for TTP and OS when the dichotomized variable was used. When calculations were done using the continuous variable, we did not observe independent significant differences. Thus, there is an apparent discrepancy in *p* values when *hTERT* is considered as a continuous versus dichotomized variable. Nevertheless, increasing pretreatment plasma concentrations of *hTERT* was significantly associated with later reduced chemotherapy efficacy, which supports the survival differences based on *hTERT* cutoffs at the median value. It may be argued that the prognostic impact by plasma *hTERT* levels not necessarily is mediated by linear changes in *hTERT* levels. It should be noted that the significant associations between *hTERT* tercils versus both TTP and survival supports our results using the dichotomized *hTERT* variable.

The DNA concentrations in our study did not seem to be influenced by either pretreatment tumor characteristics or clinical variables such as age and gender. In a recent study on 46 patients with NSCLC, van der Drift et al.[32] observed that high circulating DNA levels correlated with a worse survival, consistent with our results. Also, they did not find any relationship of DNA concentration with tumor or clinical variables. In a larger NSCLC cohort of 185 patients with NSCLC, Gautschi et al.[19] reported high baseline plasma DNA to correlate with poor survival, advanced tumor stage, lactate dehydrogenase level, and leukocytes. They also observed that high DNA levels correlated to disease progression, in agreement with the independent prognostic relevance the higher dichotomized *hTERT* level had for TTP in our study. Fournié et al.[18] examined the plasma DNA levels in 68 hospitalized patients with lung cancer, of which 46 has NSCLC. Also in this study, high circulating DNA levels are

related to poor OS and advanced disease stage. In a recently published lung cancer screening study, Sozzi et al.[33] also observed poor survival at high plasma DNA concentration.

Possible reasons for discrepancies regarding the prognostic impact of *hTERT* may be choice of patient population and sample processing. Some studies have included both small cell lung cancer and NSCLC, which differ largely in biology, treatment, and prognosis.[20,18] Most studies have included patients with both advanced and limited surgical disease,[18,19,32] whereas only patients with advanced NSCLC (IIIB and IV) were included in this study. This variability may, in part, explain why there was no correlation between *hTERT* levels and disease stage in our study. Disparities in levels of circulating DNA between studies are primarily due to protocol differences with respect to blood sample processing, DNA extraction, and DNA quantification method. Although most researchers today use quantitative polymerase chain reaction for DNA quantification, there is still a considerable potential for further standardization of sample source, sample processing, and DNA extraction methods.[10,23]

Using a dichotomized *hTERT* variable, we conclude that a high baseline level of circulating *hTERT* is a significant independent prognosticator for poor TTP and OS in advanced NSCLC. This is supported by significant negative associations between plasma *hTERT* concentrations and objective response rates, and by the association between *hTERT* tercils versus both TTP and survival. Furthermore, the *hTERT* concentration was not influenced by tumor characteristics or clinical variables. *hTERT* analysis requires a simple, noninvasive, and affordable procedure, and it may aid in therapy evaluations and follow-up of patients with NSCLC.

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